Supporting Information

Hermes et al. 10.1073/pnas.0910753106

SI Text

Animals. The colony room and the adjacent testing room were maintained at 22 ± 1 °C, 40 ± 5% relative humidity, and a 14/10-h light/dark cycle (lights on at 1000 h CST). Water and food (Rodent Diet 8604) were available ad libitum. Harlan Teklad Rodent Diet 8604 has metabolizable energy in the low normal range for a standard laboratory diet (3.1 kcal/g), grain-based (corn primary, soybean meal secondary), and low normal fat content (4.4 g/100 g diet; standard range 4.0 to 6.0 g/100 g).

Protocol Time Line. Hormonal and behavioral responses to stressors were assessed repeatedly from young adulthood through late middle age (see Fig. 5). Corticosterone stress responses were assessed at 3 and 13 months of age. Behavioral responses during an exploration stressor were assessed at 5 and 15 months of age. Behavioral responses to the every-day husbandry procedure of opening the home cage were assessed among isolates at 10 and 15 months of age. Ovarian function was quantified during late middle age (12 to 16 months of age) and anatomy at necropsy. All 12 mammary glands were palpated for mammary tumors, beginning at10 months of age and ending with necropsy at 18.8 ± 0.5 months of age when tumors were excised, diagnosed, and characterized by levels of glucocorticoid, estrogen, and progesterone receptors.

Mammary Tumor Burden. Animals were palpated for mammary tumors beginning at 9 months of age. Tumors were first palpable at 1-mm diameter, a sensitivity facilitated by location of thin flat mammary sheets between skin and the underlying muscle and bone. The diameter of palpable mammary tumors was coded in situ, measured by caliper, and converted to tumor weights based on volume of a prolate ellipsoid with the equal short axes. Tumor weight grams = {length (cm) × [width (cm)]2}/2 (1). In a cross-sectional data collection at 15 months of age, all animals were diagnosed as being tumor-free or having developed at least one tumor. The number of palpable tumors was recorded along with the total tumor burden (weight). The age for the cross-sectional study was set at 15 months to balance maximizing the opportunity tumor development against the loss of animals to the cross-sectional study because of early tumor-related deaths.

Tumor Tissue Collection And Processing. Animals were necropsied beginning at 16 months of age, if their tumors interfered with their well being (e.g., became infected or the animal cachectic, determined in consultation with a veterinarian) continuing to 22 months of age when the study ended. These criteria afforded the opportunity to validate 15 months of age as appropriate for determining tumor condition.

At 15 months of age, animals diagnosed as tumor-free or having only micromasses (0.11 ± 0.02 g; total tumor burden ≤ 1.00 g), remained tumor free and lived longer than those with tumors, (7 additional months, Log rank $\chi^2 = 24.6$, $P \leq 0.0001$). New tumors developed rarely (in 0% of rats without any palpable tumors at 15 months of age; only 2.2% of tumors found at necropsy had developed after 15 months of age). Thus, in Sprague–Dawley rats, mammary tumors are a disease of middle age; 15 months is an age by which genetic risk for different mammary pathology has been manifest (presence, benign or malignant). African-American women have a similar age-dependent risk, highest during middle age, in contrast to European-American women whose probability of cancer increases with age.

Once an animal developed a palpable diagnosable tumors, she typically lived only an additional 2 months before the disease interfered with her well being (2.0 ± 0.3 months). The average age of necropsy was 18.8 ± 0.5 months of age; no difference in age of necropsy in the two social conditions: isolates 18.9 ± 0.6 months, group-housed = 19.3 ± 0.5 months, Mantel-Cox $\chi^2 = 0.06$, $P = 0.80$).

The ovoid tumors were typically encapsulated and easily excised from each of the mammary quadrants (left and right pectoral, left and right inguinal). The total tumor burden of animals with diagnosable tumors (68% of 39 rats) was greater than those with only micromasses (17.8 ± 6.9% vs. 2.6 ± 0.4% body weight), which were equally likely to be isolated or group-housed animals (37% vs. 34%; Fisher's Exact $P = 0.99$).

Tumor tissue was fixed in 10% formalin and embedded in paraffin blocks. Sections measuring 4 μm were stained with hematoxylin and eosin (H&E), read for diagnosis by at least two surgical pathologists (Pathology Department of The University of Chicago Hospitals).

Two representative sites were chosen for 1-mm cores of tissue to be mounted in a tissue microarray, constructed using the automated Beecher tissue arrayer ATA-27 (Beecher Instrument, Inc.). TMA sections were 4 μm. After deparaffinization in xylene, slides were rehydrated through consecutive graded alcohols to distilled water. After blocking endogenous peroxidase activity, sections were heated for antigen retrieval with citrate buffer. The sections were then incubated with the primary antibodies for 1 h at room temperature (described in the main article).

The microarray technology permits >40 experimental cores per slide, so that tissues from both experimental conditions are processed identically on the same slide, and all slides can be processed simultaneously in a single batch, reducing error introduced by variation between batches of a large number of slides.

Animals with only micromasses (0.11 g) were excluded from analyses of tumor types ($n = 13$) because (*i*) most micromasses were not diagnosed and (*ii*) total micromass burden did not predict diagnosis (subset analysis of rats with diagnosed micromasses: 62% malignant diagnosis with micromass burden ≥1 gm, 38% malignant given burden <1 g; Fisher's Exact $P = 0.32$).

Corticosterone Stress Response. During the nadir of the diurnal rhythm in corticosterone (the rat equivalent of cortisol) 2-month-old rats were placed for 30 min in an unfamiliar cage scented with predator (fox) urine and the corticosterone concentration attained at the end of this stressor measured hormonal stress response. Corticosterone sampled 30 and 90 min after returning to the home cage assessed stressor recovery, defined as the drop between samples taken 30 and 90 min after stress (see Fig. 2).

When the rats became 13 months old, we modified this protocol. Other studies in our laboratory indicated that baseline levels could differ between social conditions in middle age (2), even though they did not in young animals. Moreover, prolonged recovery from stress is a hallmark of aging of the hypothalamic-pituitary-adrenal axis (3). Therefore, we added a baseline sample immediately before the imposed stressor, and measured hormonal recovery with two samples taken 60 and 120 min post-stressor.

The rise and recovery of the stress response were expressed as a change from baseline concentrations (4) (See Fig. 2). For

parametric statistical analysis, values were z scored, standardized to the time of sample collection. Recovery z-scores at 60 and 120 min after stress were correlated within individuals ($r = 0.60, P \leq 0.0003$), and therefore averaged into a single recovery z score corresponding to recovery 90 min after stress, as measured at 3 months of age.

Exploration Stress, Anxiety, and Boldness. ***Exploration arena.*** The exploration arena measured 110 cm wide × 55.2 cm high × 109.1 cm long, consisting of four sides joined to form a square. Three sides were dark sealed wood; the fourth clear Plexiglas enabling videography from an oblique angle. The arena rested on the floor; covered with a layer of wood chip cage bedding changed after each trial. A ceramic bowl in a corner served as the home base into which the animal was gently placed at the beginning of 4-min exploration trial. An overhead video camera, sensitive in dim red light, allowed recording of the rats' nocturnal activity during its "behavioral day." Behavioral sequences were analyzed using Ethovision (Noldus).

Ethogram. When rats are placed in their home bowl set in the corner of an open field, they explore with a stereotyped pattern, and exhibit species-typic behaviors indicating anxiety and vigilance as well as boldness and curiosity. Some rats take a long time to leave their home bowl, and then move slowly along one wall (from one to five rat body lengths), returning home before going out again along the same or adjacent wall. Other rats will then round the next corner, return, and explore around the corner on the opposite wall. Less than one-third of rats will eventually move around all four sides, with sorties away from the wall, cutting corners or completely traversing the open field. We created a composite score to quantify the animals' responses to the open field, measuring the balance of exploration and vigilance, a behavioral stress response.

Exploration behaviors were quantified with four measures: latency to move from home base, distance traveled (measured by the number of grid lines crossed, new areas entered, and thigmotaxis (time in contact with the wall). A confirmatory oblique factor analysis of the z-scores substantiated averaging the z-scores into a single score: latency to leave home bowl (−0.94), new areas entered (+0.93), distance covered (+0.93), and time in contact with the walls (+0.71); Eigen value = 3.1, Bartlett's Chi Square = 114.6, proportion of variance = 0.78, $P \leq 0.0001$).

Anxiety. The frequencies of anxiety behaviors were summed and expressed as a z score, standardized to the mean for each age of testing: freezing, piloerection, urination, or defecation outside the toilet area, stereotyped grooming).

Boldness was quantified similarly by summing bold behaviors and expressing as a z score (standardized to the mean for each age of testing): rearing on hind legs, with or without a supporting paw, standing, lack of hesitant gait, progression through the four stages of exploration.

Exploration stress. Average to low levels of exploration behaviors were validated as an anxiety measure and high exploration levels were validated as a boldness measure. Animals who explored with average to below average levels (≤ +0.25 z score) also exhibited a variety of anxiety and vigilance behaviors, increasing linearly with less exploration (piloerection, freezing, defecation/urination in the field, and stereotypical grooming; $r = -0.51, P \leq 0.0001$).

Females with above average exploration scores exhibited bold behaviors (rearing on their hind legs, standing supported by a vertical surface, speed (0, ≤ 1ft/s, > 1 ft/s), and progress through the stages of species-typic exploration ($r = 0.64$, $P \leq 0.0001$). Nonetheless, among females with below average exploration, exploration did not have a linear relationship with boldness behaviors.

Therefore, the inverse of the exploration factor score was used as an indicator of Exploration Stress, associated within individual rats at mid to high ranges with anxiety and vigilance, and in the low range boldness and curiosity.

Vigilance During Every-Day Stressors. Standard husbandry procedures are routine rodent stressors (5). When the home cage is opened daily to check food and health, or to obtain a vaginal cytology sample, some rats cower in the back, while others quickly come to the front and even rise up to inspect the investigator. These individual differences in vigilance and behavioral stress reactivity to repeated every-day stressors were quantified by leaving the cage open for up to 5 min and recording the latency for the rat to emerge from the back and latency to stand at the front with a paw on the rim.

Reproductive Senescence. ***Dynamic ovarian function.*** Reproductive senescence in the rat is indicated by the onset of irregular cycles and, in some animals, entry into a state of tonic unopposed estrogen (constant estrus), a dynamic temporal pattern, which must be measured noninvasively with vaginal cytology following a well-established protocol. From daily vaginal lavages (mid-dark dark-phase), the changing proportion of cornified epithelial cells, nucleated epithelial cells, and leukocytes indicates estrous cycle phase (6, 7). The number of periovulatory surges of estrogen (i.e., during proestrus) included those that began a complete cycle (marked by two successive proestrous days) as well the proestrous day beginning a cycle that was not completed by the end of the observation period. Estrogenization level was quantified by percent of 14 days with only nucleated or cornified vaginal epithelial cells, a well-established bioassay for estrogen level (8).

Ovarian anatomy. In middle-aged animals, we determined which animals had reached the irreversible state of estropause, that is, ovarian senescence. There are two patterns of ovarian senescence in this species—one of irregular cycles, followed by constant estrus, and a return to irregular cycles. Other females simply maintain irregular cycles during middle age. Therefore, months of continuous vaginal cytology records are needed to characterize which aging pattern the animal takes and whether irregular cycles represent irreversible ovarian senescence. Here, we needed a single measure close in time to the assessment of mammary tumor burden that could unequivocally determine the state of ovarian senescence—the anatomy of the ovary. Moreover, it was known that social isolation accelerated reproductive senescence measured dynamically with daily vaginal smears (9); here, we sought to verify this by quantifying ovarian function directly.

For animals necropsied at 16 months of age, ovaries were weighed, formalin fixed and paraffin embedded and serial sections (10 μm) were stained with hematoxylin and eosin. The presence of tertiary follicles, ova and recent or involuting corpora lutea confirmed ovulation and steroidally active ovarian tissue. Secondary and atretic follicles were also identified (10).

1. Simpson-Herren L, Lloyd HH (1970) Kinetic parameters and growth curves for experimental tumor systems. *Cancer Chemother Rep* 54:143–174.
2. Hermes GL, Rosenthal L, Montag A, McClintock MK (2006) Social isolation and the inflammatory response: Sex differences in the enduring effects of a prior stressor. *Am J Physiol Regul Integr Comp Physio* 290:R273–282.
3. Sapolsky RM (1996) Stress, glucocorticoids, and damage to the nervous system: The current state of confusion. *Stress* 1:1–19.
4. Vahl TP, et al. (2005) Comparative analysis of ACTH and corticosterone sampling methods in rats. *Am J Physiol Endocrinol Metab* 289:E823–828.
5. Cavigelli SA, et al. (2005) Frequent serial fecal corticoid measures from rats reflect circadian and ovarian corticosterone rhythms. *J Endocrinol* 184:153–163.
6. Gans SE, McClintock MK (1993) Individual differences among female rats in the timing of the preovulatory LH surge are predicted by lordosis reflex intensity. *Horm Behav* 27:403–417.

7. LeFevre J, McClintock MK (1988) Reproductive senescence in female rats: A longitudinal study of individual differences in estrous cycles and behavior. *Biol Reprod* 38:780–789.
8. Nequin LG, Alvarez J, Schwartz NB (1979) Measurement of serum steroid and gonadotropin levels and uterine and ovarian variables throughout 4 day and 5 day estrous cycles in the rat. *Biol Reprod* 20:659–670.
9. LeFevre J, McClintock MK (1991) Isolation accelerates reproductive senescence and alters its predictors in female rats. *Horm Behav* 25:258–272.
10. Hermes GL, McClintock MK (2008) Isolation and the timing of mammary gland development, gonadarche, and ovarian senescence: Implications for mammary tumor burden. *Dev Psychobiol* 50:353–360.